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Selectively replicating adenoviruses targeting deregulated E2F activity are potent, systemic antitumor agents

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Summary

Summary Significance Introduction Results Discussion Experimental procedures Referenc

We have engineered a human adenovirus, ONYX-411, that selectively replicates in human tumor cel
but not normal cells, depending upon the status of their retinoblastoma tumor suppressor protein (pR
pathway. Early and late viral gene expression as well as DNA replication were significantly reduced
functional pRB-pathway-dependent manner, resulting in a restricted replication profile similar to tha
nonreplicating adenoviruses in normal cells both in vitro and in vivo. In contrast, the viral life cycle i
tumor cell killing activity of ONYX-411 was comparable to that of wild-type adenovirus following
infection of human tumor cells in vitro as well as after systemic administration in tumor-bearing
animals.

Significance

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Therapeutic agents with enhanced efficacy and strong tumor-cell selectivity are needed to treat huma cancers. One novel strategy is to use selectively replicating human adenoviruses, which amplify and spread throughout the tumor tissue, resulting in direct lysis of an expanded population of cancer cells We have engineered ONYX-411 to take advantage of the disruption of the pRB-pathway, one of the hallmarks of human cancer. Because of its strict dependency upon the functional status of the pRB-pathway, ONYX-411 has a high therapeutic index and is effective as a systemic antitumor agent in animal models of human cancer. In addition, ONYX-411 gene expression is highly tumor cell selecti making it an ideal delivery vehicle that will express therapeutic transgenes specifically at the tumor s

Introduction

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Human adenoviruses normally infect, propagate, and lyse their host cells to release newly generated progeny virus. Although these properties are ideal for killing human cancer cells, virus replication m be restricted to tumor and not normal cells and tissues. Mutant adenoviruses have now been develop that are restricted from efficiently completing their life cycle in normal cells, but selectively propaga in and lyse tumor cells. In multiple clinical trials and animal models of human cancer, selectively replicating adenoviruses have shown varying degrees of antitumor efficacy.

Different approaches have been taken to restrict human adenovirus replication in normal cells (Kirn al., 2001 ☒; McCormick, 2001 ☒). Originally, the recognition that both tumor cells and human adenoviruses inactivate the p53 and pRB tumor suppressor protein functions led to the strategy for engineering adenoviruses to selectively propagate in human cancer cells based upon their p53- or pR pathway status (Bischoff et al., 1996 ☒; Fueyo et al., 2000 ☒). This strategy is based upon the concep that the loss of pRB- or p53-pathway functions in tumor cells should permit replication of E1A or E1 gene mutant viruses, respectively (Bischoff et al., 1996 ☒; McCormick, 2001 ☒).

Following adenovirus infection, E1A is the first viral gene product expressed, and is required for efficient transcription of all other viral early gene regions (Berk, 1986 ☒; Berk et al., 1979 ☒; Jones a Shenk, 1979 ☒). E1A also promotes S phase entry, partly through binding and inactivation of pRB ar its related family members (Bayley and Mymryk, 1994 ☒; Howe and Bayley, 1992 ☒; Howe et al., 1990 ☒; Wang et al., 1991, 1993 ☒). The adenovirus E1B region encodes two major polypeptides, E1 19K and E1B-55K, both of which function to inhibit p53-mediated apoptosis of virally infected cells (Debbas and White, 1993 ☒; Lowe and Ruley, 1993 ☒; White, 1995 ☒). E1B-55K directly binds to p! and represses its transactivation activity (Teodoro and Branton, 1997 ☒; Teodoro et al., 1994 ☒; Yew and Berk, 1992 ☒; Yew et al., 1994 ☒), and is required in complex with the adenovirus E4 gene regic product, E4orf6, to target p53 for degradation (Moore et al., 1996 ☒; Nevels et al., 1997 ☒; Querido ε al., 1997 ☒; Steegenga et al., 1998 ☒).

Like E1A and E1B, the adenoviral E4 gene region is expressed early after infection and is an essenti component of the viral life cycle. The E4 gene region encodes several products whose functions affe the nucleocytoplasmic shuttling of viral RNAs, efficient synthesis and processing of early and late vi proteins, the induction of apoptosis, and cellular transformation. The E4 gene products function in concert with E1A and E1B to create a cellular environment permissive for efficient expression and

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processing of viral gene products and ultimately a productive virus infection.

ONYX-015 (*dl*1520), an E1B-55K gene-deleted human adenovirus that cannot inactivate p53 (Barker and Berk, 1987⊞), was the first therapeutic virus shown to preferentially propagate in cancer cells based upon their p53 functional status (Bischoff et al., 1996⊡; Ries et al., 2000⊞; Yang et al., 2001⊡). ONYX-015 is currently in phase III clinical trials in combination with standard chemotherapy for the treatment of patients with recurrent cancers of the head and neck.

pRB plays an essential regulatory role in mediating appropriate cellular responses to extracellular stimuli (Bartek et al., 1997⊡; Larminie et al., 1998⊡; Weinberg, 1995⊡). pRB functions as a transcriptional repressor to negatively regulate S phase entry by directly binding to and repressing the transactivation activity of the E2F family of transcription factors (Dyson, 1998⊡; Harbour and Dean, 2000⊡). By binding to their promoters, E2Fs regulate the expression of a diverse set of genes involved in nucleotide metabolism, DNA replication, cell cycle control, apoptosis, and differentiation (Helin, 1998⊡; Johnson and Schneider-Broussard, 1998⊞; Muller and Helin, 2000⊡). DNA tumor viruses target pRB family members, resulting in elevated E2F activity and efficient S phase entry, the cell cycle phase most permissive for viral DNA replication and progeny production (Dyson et al., 1989⊡; Moran, 1993⊡). For human adenovirus, two noncontiguous domains of E1A, conserved region 1 (CR1, low affinity) and conserved region 2 (CR2, high affinity), are required to mediate pRB binding and inactivation (Dyson et al., 1992⊡; Fattaey et al., 1993⊞). In concert with E1A function, human adenoviruses further target and direct E2F onto the viral E2 promoter through direct complex formation between the E4 gene region product, E4orf6/7, and E2F (Cress and Nevins, 1994⊞; Helin and Harlow, 1994⊡; Neill et al., 1990⊞; Neill and Nevins, 1991⊡; Obert et al., 1994⊡; Raychaudhuri et al., 1990 ⊞).

Human adenoviruses carrying mutations within the E1A-CR2 domain have been tested in the laboratory as potential anticancer agents (Fueyo et al., 2000⊞; Heise et al., 2000⊡). Although these virus mutants can efficiently replicate in both dividing and nondividing tumor cells, their selectivity in normal cells and safety in animal models has not been demonstrated. Another adenovirus mutant has recently been described that targets both the p53 and pRB pathways (Ramachandra et al., 2001⊡). Similarly, the mechanism providing its tumor selectivity or in vivo utility as a safe and effective systemic anticancer agent has yet to be demonstrated.

An alternative approach for developing tumor cell selectivity has been the use of tissue- or tumor-specific promoters to control the expression of the adenovirus early gene regions E1A, E1B, E2, or E4 (Brunori et al., 2001⊡; Doronin et al., 2001⊡; Fuerer and Iggo, 2002⊞; Hallenbeck et al., 1999⊞; Hernandez-Alcoceba et al., 2000⊞; Kurihara et al., 2000⊞; Rodriguez et al., 1997⊡; Yu et al., 1999a, 1999b⊞). By definition, tissue-specific promoters lack tumor cell selectivity. Another limitation of this approach is that the selected promoter may not be active in all tumor cells derived from the particular tissue type for which it was chosen. This may be due to heterogeneity in the genetic alterations and differentiated state of many tumors, both of which could influence the activity of tissue-specific promoters. Furthermore, the mechanism used to establish selectivity may limit their broad utility to treat cancers of different tissue types.

Here we present a safe and systemically effective human adenovirus, ONYX-411, that selectively

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replicates in a broad array of human tumor cell types based upon their defective pRB-pathway status. The pRB-pathway is disrupted in nearly all human cancers (Hanahan and Weinberg, 2000☒; Sherr, 2000☒), and hence, the oncolytic activity of ONYX-411 is not limited to tumors of a particular tissue type. ONYX-411 contains a deletion within the E1A-CR2 region, and its pRB-selectivity was further enhanced by replacement of the viral E1A and E4 promoter regions with the human E2F1 gene promoter. The combination of these attributes was essential for selective viral gene expression, viral DNA replication, and tumor cell killing both in vitro and in vivo.

Results

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Construction and characterization of virus mutants

Our goal was to develop a human adenovirus that can selectively replicate in human cancer cells based upon disrupted pRB-pathway function. In order to generate pRB-pathway selectivity, we chose to regulate the expression of the E1A and E4 gene products using the pRB-responsive human E2F1 promoter. This promoter was selected because: (1) it is subject to autoregulatory control in a cell cycle-dependent manner via four consensus E2F DNA binding sites; (2) its activity closely correlates with the pRB-pathway status of the cell; (3) the E2F DNA binding sequences present within the promoter mediate both repression by pRB and stimulation by wild-type, but not CR2 mutants of E1A; and (4) it has been used to direct pRB-dependent, tumor-selective gene expression in vivo, which differentiates between normal and aberrant cell cycle states (Johnson et al., 1994☒; Neuman et al., 1994☒; Parr et al., 1997☒).

To test pRB-responsiveness, we constructed plasmids representing the left end of the *dl*922/947 (E1A-ΔCR2) human adenovirus genome (Whyte et al., 1989☒) in which the E1A promoter region was replaced with the previously characterized wild-type or mutated alleles of the human E2F1 promoter (Parr et al., 1997☒) (Figures 1 and 2). We chose to use the *dl*922/947 mutation of E1A for our studies since it had been shown that E1A-ΔCR2 alleles were not able to overcome pRB-mediated repression of the E2F DNA binding sequences present within this promoter. In addition, human adenoviruses carrying mutations within this E1A domain have previously been tested for their selective replication properties in cancer cells (Fueyo et al., 2000☒; Heise et al., 2000☒). To demonstrate pRB-regulated E1A gene expression, C33-A cervical carcinoma cells (pRB mutant) were cotransfected with these constructs along with expression vectors containing either wild-type pRB or pRB-ΔCdk, an allele of pRB mutated in all potential CDK phosphorylation sites (Leng et al., 1997☒). pRB-ΔCdk is a constitutively active allele of pRB that is not subject to cell cycle regulation. This allele was used to distinguish between direct inactivation of pRB by E1A versus inactivation of pRB by Cdk-mediated phosphorylation as a consequence of E1A-induced S phase entry. Ectopic expression of either pRB or pRB-ΔCdk resulted in significant repression of the human E2F1 promoter and E1A expression (Figure 2A, lanes 2 and 3). Importantly, pRB-mediated repression was dependent upon intact E2F DNA binding sequences, as the promoter construct mutated only in the E2F DNA binding sites was insensitive to pRB expression (Figure 2A, lanes 5 and 6). Interestingly, the highest levels of expression were also observed when E1A was regulated by the wild-type E2F1 promoter (Figure 2A, compare lane 1 to lanes 4 and 7). This was in accordance with previous observations regarding the relative strength of the E2F1 promoter (Parr et al.,

1997☒). It is important to note that the endogenous E1A promoter was also responsive to pRB-ΔCdk expression (Figure 2A, lane 9). This may be due to either direct regulation by pRB through the E2F DNA binding sites in the endogenous E1A promoter or as a consequence of cell cycle arrest induced by pRB-ΔCdk expression.

Figure 1. Diagram of viruses examined throughout the course of this study

Mutations specific to each virus are indicated by the affected region. The presence of a complete deletion is represented by Δ, whereas the minimal deletion of the E1A-CR2 domain from nucleotides 922 to 947 is indicated by ΔCR2. All viruses were created on an Ad5, dl309 background, which is characterized by a deletion within the E3B region that removes the E3-10.4K, 14.5K, and 14.7K immunoregulatory genes. ONYX-015 (dl1520) carries a deletion of the E1B-55K gene, whereas dl922/47 contains the minimal E1A-CR2 deletion. Unique restriction sites were engineered into the dl922/47 backbone such that the E1A and/or E4 promoters could be selectively replaced with the human E2F1 promoter. In the case of the E1A promoter, HindIII and BamHI sites were generated at nucleotides 377 and 538, respectively. The E4 promoter was replaced through the use of newly created XhoI and SpeI sites at nucleotides 35,576 and 35,815, respectively. Importantly, core enhancer and DNA packaging elements remained intact at both ends of the viral genome.

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Figure 2. The human E2F1 promoter is responsive to the status of pRB and E1A
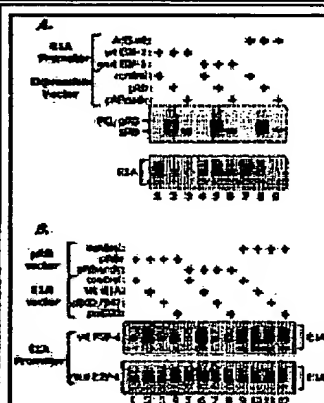
A: The E1A promoter was either left intact (Ad5) or replaced with the human E2F1 promoter, wild-type (wt) or mutated (mut) in the E2F/DP binding sites, in a plasmid containing nucleotides 22 to 5,790 of the Ad5 genome. Each plasmid carried an additional deletion within the E1A-CR2 domain from nucleotides 922 to 947. The promoter-specific plasmids were examined for pRB-responsiveness by cotransfection assays with a vector expressing wt pRB or pRB-ΔCdk in C33-A cells, a tumor cell line that is deleted for RB1. Lysates were harvested 48 hr posttransfection and analyzed for pRB and E1A expression by immunoblotting. B: The same plasmids in A were cotransfected with an additional plasmid that constitutively expressed a truncated version of E1A that was wt, deleted (dl922/947), or mutated (pm928) within the E1A-CR2 domain. These constitutively expressed forms were truncated at their C termini so that they could be distinguished from the reporter, E2F1 promoter-driven E1A. Lysates were harvested and analyzed for reporter-specific E1A expression as in A. The results are representative of two independent experiments.

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These results demonstrated pRB-mediated regulation of the E2F1 promoter in the context of the adenoviral genome as measured by E1A expression. We chose to use an E1A-ΔCR2 allele for our studies since it is possible that any residual wild-type E1A expression could inactivate pRB, thereby circumventing regulation of the E2F1 promoter. To demonstrate this possibility, experiments similar to those in Figure 2A were conducted, except that an additional plasmid was cotransfected that contained either wild-type E1A or E1A-ΔCR2 mutant alleles ($dl922/947$ or $pm928^{C \rightarrow G}$). Expression of exogenous wild-type E1A (Figure 2B, lanes 2 and 6), but not E1A-ΔCR2 mutants (Figure 2B, lanes 3, 4, 7, and 8), was capable of inactivating pRB- and pRB-ΔCdk-mediated repression of the E2F1 promoter. Moreover, this inactivation by wild-type E1A was dependent upon intact E2F DNA binding sites within the E2F1 promoter. These results demonstrated that: (1) the E2F1 promoter was active in the context of the left end of the adenoviral genome, and (2) its activity was responsive to pRB-pathway status only in combination with ΔCR2 mutant alleles and not wild-type E1A. Based on these results, we modified the E1A-ΔCR2 mutant adenovirus, $dl922/947$ (Whyte et al., 1989⊞), by replacing viral regulatory sequences within the E1A (ONYX-150), E4 (ONYX-410), or both gene regions (ONYX-411) with the human E2F1 promoter (Figure 1).

Viral gene expression, induction of S phase entry, and viral DNA replication

To examine the link between RB status and viral gene expression, primary mouse embryonic fibroblasts (MEFs) from isogenically matched $RB^{+/+}$ and $RB^{-/-}$ littermates were infected with the panel of adenoviruses shown in Figure 1. Regulation of the E1A region (ONYX-150) of human adenovirus by the E2F1 promoter resulted in a partial repression of E1A protein expression in $RB^{+/+}$ but not $RB^{-/-}$ MEFs (Figure 3). The most significant reduction in E1A expression levels, however, was observed when the E2F1 promoter regulated both the E1A and E4 regions (ONYX-411). This reduction in E1A levels was only observed in the $RB^{+/+}$ and not the $RB^{-/-}$ MEFs and was independent of their proliferation status (Figure 3, lanes 17–21). Interestingly, elevated E1A levels were observed in MEFs infected with ONYX-410 (see Discussion). These results indicate that pRB-regulated E1A gene expression was best achieved with ONYX-411, where the E2F1 promoter controlled both the E1A and E4 gene regions.
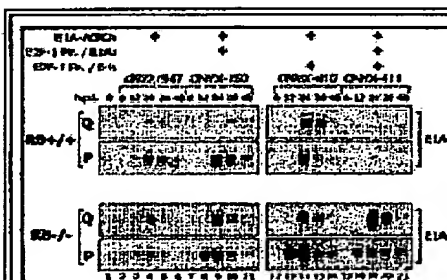
Figure 3. E2F1 promoter-regulated adenoviruses are pRB-responsive

Primary mouse embryonic fibroblasts (MEFs) from isogenically matched $RB^{+/+}$ and $RB^{-/-}$ littermates were propagated under proliferating (P) or quiescent (Q) growth conditions and infected with $dl922/47$, ONYX-150, ONYX-410, or ONYX-411. A "+" sign indicates the alterations specific to each virus. Cell lysates were generated at various hours postinfection (hpi) and examined

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for E1A protein expression by immunoblot analysis. The results
are representative of two independent experiments.

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E2F activity is deregulated in most human cancers as a result of pRB-pathway disruption. Regulated expression of the E1A and E4 gene regions by the E2F1 promoter should, therefore, be preferentially restricted to human tumor cells and not normal cells. To test this directly, early and late viral protein expression was measured following infection of a panel of human tumor cell lines and normal proliferating (SAEC-P) or contact inhibited (SAEC-CI) primary human lung epithelial cells. We examined six different tumor cell lines of lung (H1299, H2009, H460, and H2172), pancreatic (MiaPaCa), and breast (MDA-MB-231) tissue origin, all of which were defective in their pRB-pathway status (Figures 4A–4C and data not shown; see Experimental Procedures for the status of pRB-pathway components in each cell line). E1A protein levels were significantly lower in primary human lung epithelial cells (SAEC-P and SAEC-CI) infected with either ONYX-150 or ONYX-411, where E1A was regulated by the human E2F1 promoter (Figure 4A). This was in contrast to the E1A levels detected in normal cells following infection with all of the other viruses where E1A was controlled by its endogenous promoter (dl309, dl922/947, ONYX-015, and ONYX-410) (Figure 4A). Importantly, E1A expression was at wild-type or enhanced levels in tumor cells infected with all viruses (Figure 4A). Similar to results obtained after infection of MEFs, E1A expression was markedly higher and sustained following infection of primary human cells with ONYX-410 (see Discussion). Expression of the E4 gene region products, E4orf6 and E4orf6/7, was significantly reduced in normal cells infected with ONYX-410 and ONYX-411, where E4 transcription was controlled by the human E2F1 promoter (Figure 4B). This was in contrast to the near wild-type expression kinetics and levels of E4orf6/7 detected in tumor cells infected with all viruses (Figure 4B). We were unable to readily detect E4orf6 expression in infected tumor cells in these studies. Taken together, the results in Figures 4A and 4B indicate that E2F1 promoter-regulated transcription of the E1A and E4 viral gene regions resulted in a significant repression of early viral gene expression in normal versus tumor cells.
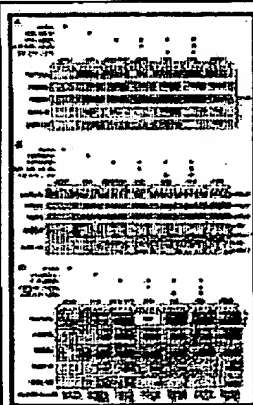
Figure 4. E2F1 promoter-driven adenoviruses demonstrate tumor-selective gene expression in infected human cells

Human tumor cell lines (MiaPaCa, MB231, H1299) or primary small airway epithelial cells that were either proliferating (SAEC-P) or arrested through contact inhibition (SAEC-CI) were infected with dl312, dl309, ONYX-015, dl922/47, ONYX-150, ONYX-410, or ONYX-411. A "+" sign indicates the alterations specific to each virus. At various times postinfection (T1–T5), cell lysates were generated and analyzed by immunoblotting for their early and late gene expression profiles. The hours postinfection (hpi) represented by T1–T5 were as follows: MiaPaCa (14, 24, 36, 50, and 64 hpi), MB231 (12, 25, 36, 47, and 73 hpi), H1299 (12, 25, 37, 48, and 61 hpi), and SAEC-P/-CI (6, 12, 24, 36, and 48 hpi). A–C: Early gene expression patterns are demonstrated for E1A (A),

E4orf6 (**B**), and E4orf6/7 (**B**). In C, late gene expression patterns are shown for hexon (h), penton (p), and fiber (f). In all cases, equal loading was demonstrated by immunoblot analysis for β-actin (data not shown). In addition, we performed quantitative PCR analysis to examine adenovirus DNA levels at 12, 24, 36, and 48 hpi in SAEC-CI cells. Samples were analyzed in duplicate and are represented as the fold increase in signal relative to that detected with dl312 at 12 hpi. ND, not determined. These results are representative of at least two independent experiments in the primary SAECs and once in 6 different human tumor cell lines, three of which are shown.

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Adenoviral early gene expression is required for induction of cellular S phase entry, viral DNA replication, and subsequent expression of late viral messages and capsid proteins. Therefore, we next examined how each of these steps was affected in the different E2F1 promoter-regulated viruses. To examine how viral early gene expression affects the host cell's division cycle, we analyzed the cell cycle profile of synchronized, contact-inhibited SAECs following infection with the panel of viruses used above. Cells were harvested at various times postinfection and analyzed by flow cytometry to determine the fraction of cells in the G1, S, or G2/M phases of the cell cycle. As compared to wild-type virus (dl309) infected cells, a significantly lower fraction of normal cells infected with ONYX-150, ONYX-410, or ONYX-411 were in S phase at various times postinfection (Figure 5). In contrast, the fractions of ONYX-015- and dl922/947-infected cells in S phase were similar to wild-type-infected cells (Figure 5). As determined by quantitative PCR analysis, the levels of viral DNA replication closely reflected both the time of entry and fraction of cells in S phase following infection with each of the viruses (Figure 4C). These studies demonstrate that ONYX-411 (regulation of both E1A and E4 gene regions by the E2F1 promoter) was severely attenuated in its ability to induce S phase entry and to replicate its DNA in normal cells.
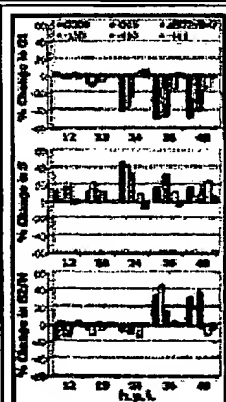
Figure 5. E2F1 promoter-driven adenoviruses are restricted in their ability to promote S phase entry

Primary human small airway epithelial cells (SAECs) were arrested by prolonged contact inhibition and infected with the indicated viruses. Cells were collected at various hpi and analyzed by flow cytometry. At a given time postinfection, the percentage change in the G1, S, and G2/M phases of the cell cycle that resulted following infection with each of the replication-competent adenoviruses is represented relative to the percentage of each phase following infection with the replication-deficient, dl312 adenovirus (e.g., % S phase [dl309–dl312] at equivalent hpi). These results are representative of at least two independent experiments.

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Adenovirus late protein expression is dependent upon viral DNA replication. Therefore, we expected that the attenuated replication of the E2F1 promoter-regulated viruses in normal cells would result in decreased late viral protein expression. As compared to infected tumor cells, remarkably low levels of the viral capsid proteins hexon (h), penton (p), and fiber (f) were detected in contact inhibited normal lung epithelial cells infected with ONYX-150, ONYX-410, and ONYX-411 (Figure 4C). Although at lower than wild-type levels, capsid protein expression was observed in proliferating normal cells (SAEC-P, Figure 4C) infected with ONYX-150 and ONYX-410 (E2F1 promoter driving E1A or E4, respectively). Taken together, these results indicate that the combined regulation of the E1A and E4 gene regions (ONYX-411) resulted in the greatest inhibition of both cellular and adenoviral DNA replication as well as late viral protein expression in normal human cells, regardless of their proliferative status (Figures 4C and 5, and data not shown).

In vitro and in vivo efficacy of pRB-regulated adenoviruses
We next sought to determine the cell killing properties of the pRB-dependent viruses in infected normal and tumor cells. A quantitative cell viability assay was used to measure the extent of cell killing by each virus (see Experimental Procedures). The cell lines examined were selected to represent a broad range of tumor tissue types including carcinomas of the lung (H1299, H2009, H460, H522, H520, and A549), colon (SW620), pancreas (MiaPaCa), prostate (DU145), breast (MB231, MB468), cervix (C33-A), and head and neck (HLaC), as well as osteosarcomas (U2OS). The normal cells tested also represented a broad range of primary epithelial cell types, including lung (SAEC, small airway; or BEC, bronchial epithelial cells), liver hepatocytes, prostate (PrEC), breast (MEC), and kidney (REC), as well as endothelial cells (MVEC, microvascular endothelial cells derived from either lung or dermis). In the panel of tumor cell lines examined, all of the E2F1 promoter-driven viruses demonstrated a cell killing capacity that was comparable to wild-type adenovirus (Figure 6 and data not shown). In contrast, the E2F1 promoter-driven viruses were markedly attenuated in their ability to kill all normal cells tested, independent of the cellular proliferation status. ONYX-411's cytolytic activity in normal cells was comparable to the replication-defective adenovirus, dl312, and was approximately 100- to >1000-fold lower than wild-type adenovirus, dl309. These results demonstrate that ONYX-411 had the greatest degree of selective cell killing activity.
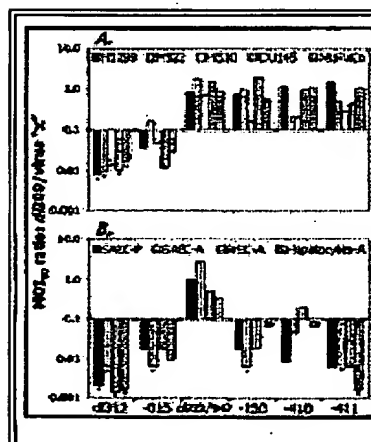

Figure 6. Selective tumor cell killing by E2F1 promoter-regulated adenoviruses

Cells were plated at varying densities and infected the following day at varying MOIs with the indicated viruses. A and B: At selected days postinfection, cell viability was determined by MTT analysis (see Experimental Procedures). For each tumor cell line and normal cell type, a minimum of two independent assays was each performed in quadruplicate. A representative subset of this data is shown for three human, matched tissue types of tumor (A) and normal (B) cell origin. For lung, 3 non-small cell lung carcinomas (H1299, H522, and H520) versus normal proliferating (SAEC-P) and contact inhibited (SAEC-A)

small airway epithelial cells are shown. The DU145 prostate carcinoma versus its normal, arrested epithelial cell counterpart, PrEC-A, is shown. Normal, arrested human hepatocytes were used as a tissue comparison for the pancreatic carcinoma cell line, MiaPaCa. MOI_{50} is defined as that MOI which results in 50% cell killing at a particular stop point. The data is presented as the MOI_{50} for each virus relative to that of $dl309$, where low MOI_{50} ratios are indicative of attenuation. In some instances (*), the degree of attenuation in cell killing by certain viruses is so great that the true MOI_{50} can not be determined. Therefore, in these cases, the extent of attenuation is actually greater than depicted. ND, not determined.

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The systemic delivery of replicating human adenoviruses, even those modified for tumor-selective replication, has previously been shown to cause dose-limiting toxicity in mice. This toxicity is associated with liver necrosis and is thought to be a result of the administered dose of adenoviral coat proteins, viral early gene expression, viral replication, and/or de novo late gene expression. The host immune response may also play a role in mediating this dose-limiting toxicity. To evaluate the safety profile of the E2F1 promoter-driven viruses in vivo, the maximum tolerated single dose of each virus was determined following intravenous (i.v.) administration in Balb/c mice. Survival, weight loss, and serum levels of the liver enzymes aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were used as measures of toxicity following administration of increasing doses of virus.

One hundred percent survival and no increases in serum AST/ALT levels were observed in all of the vehicle control and experimental treatment groups (n = 10/cohort) at the 6×10^8 plaque forming unit (pfu) dose 48 hr (day 3) after virus administration (Figure 7A). Toxicities were first observed at the 2×10^9 pfu dose. Weight loss preceded death, which occurred as early as day 4 following wild-type ($dl309$) virus administration. At this dose, only 40% of the mice treated with wild-type virus survived to day 7, 30% survived to day 12 following ONYX-150 administration, and 80% survived to day 7 following ONYX-410 dosing. In contrast, all mice survived when treated at the 2×10^9 dose of either $dl922/947$ or ONYX-411 for the duration of the study (n = 10/cohort; data not shown).

Figure 7. ONYX-411 results in reduced systemic toxicity and increased survival following intravenous (i.v.) administration

A: BALB/c mice were injected with a single i.v. dose of increasing amounts (pfu) of the indicated viruses. On day 3, 48 hpi, mice (n = 3) were analyzed for their serum AST/ALT enzyme levels. Two independent studies were performed at the 4×10^9 dose, with the number of associated deaths at day 7 indicated. ND, not determined. B: Livers were harvested
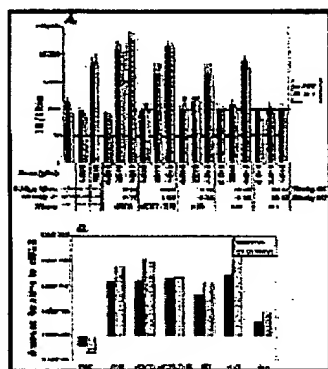
on day 3 from mice (n = 3) injected at the 4×10^9 dose and analyzed for both adenoviral DNA and E1A-13S mRNA levels by quantitative PCR analysis. The resulting signals are represented relative to that detected with dl312-injected mouse liver samples (e.g., dl309 DNA signal/dl312 DNA signal).

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At the single i.v. dose of 4×10^9 pfu, ONYX-411 was the only replicating virus that did not result in increased serum AST/ALT levels on day 3 (Figure 7A). At this dose, no mice survived beyond day 7 when treated with either wild-type or ONYX-410, and only one of twenty dl922/947-treated animals survived beyond day 7 (Figure 7A). In contrast, the ONYX-411 treated cohorts demonstrated a delayed toxicity beginning on day 8. ONYX-411 treated animals did not survive beyond day 12 in study 1, whereas all of the ONYX-411 treated mice survived to day 18 (duration of study) in the second study. Additional studies demonstrated that the ONYX-411-induced toxicity was always significantly lower in both the time of onset and the number of affected animals as compared with all other replicating viruses tested.

Adenovirus preparations often contain a substantial proportion of defective particles. It is important to note that the lower toxicities observed with the pRB-regulated viruses were not due to the administration of reduced virus particle numbers as the particle:pfu ratios for all viruses tested were within ~0.5- to 8-fold of each other. In fact, more particles per dose were delivered for all of the pRB-regulated viruses compared to wild-type virus, dl309 (data not shown). Therefore, we sought to determine if the reduced toxicity inherent to ONYX-411 correlated with viral replication and gene expression levels. Livers were harvested from mice (n = 3) on day 3 following administration of a single i.v. dose of 4×10^9 pfu and analyzed by quantitative PCR. The extent of E1A gene expression as well as adenoviral DNA replication closely mirrored the observed increases in serum AST/ALT levels at equivalent times postinjection for each of the adenoviruses (compare Figures 7A and 7B, and data not shown). As compared to wild-type virus (dl309), ONYX-411 exhibited the greatest attenuation in viral DNA replication (~50-fold) and E1A gene expression (~100-fold) in normal mouse livers in vivo (Figure 7B). The improved safety profile of ONYX-411 was further demonstrated in primary murine hepatocytes propagated in vitro, where it was significantly impaired for cell killing (>10-fold) as well as E1A and late protein expression when compared to wild-type virus (data not shown).

Taken together, these results demonstrated that ONYX-411 had the greatest selectivity index in vitro as well as the best safety profile in vivo when compared to the other adenoviruses tested. We next

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evaluated the systemic antitumor efficacy of ONYX-411 in nude mice (n = 10/cohort) bearing subcutaneous (s.c.) C33-A human xenograft tumors. A total dose of 2×10^9 pfu was administered intravenously as a daily regimen of 4×10^8 pfu for five consecutive days. Both ONYX-411 and wild-type virus (dl309) demonstrated a significant survival advantage relative to vehicle control (p = 0.03 and 0.005, respectively), and resulted in equivalent numbers of complete regressions (CRs: n = 3/10; Figure 8A). Similar levels of ONYX-411 and dl309 virus replication were observed in tumor samples by in situ hybridization at 60 and 66 days posttreatment, respectively (Figure 8B). In two independent studies using the C33-A cervical cancer xenograft model, increasing the dose of ONYX-411 by approximately 2-fold (3.75×10^9 pfu total, administered as 7.5×10^8 pfu on days 1–5) resulted in a comparable survival advantage at day 70 (range: 60%–70% of mice) without increased toxicity (Figure 8A). In these studies, cure rates ranged from 25%–60% (Figure 8A). Subsequent studies have also demonstrated that ONYX-411 can be administered systemically in tumor bearing, immunocompromised mice (Balb/c genetic background) at doses up to a total of 5×10^9 pfu (1×10^9 pfu administered on days 1–5) with no increased toxicity. This dose is 2-fold higher than the previously reported tolerated dose for ONYX-015 (Heise et al., 1999⊞) (data not shown).
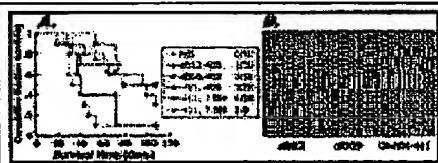

Figure 8. Tumor efficacy and evidence of viral replication following i.v. administration of ONYX-411 in mice bearing C33-A human tumor xenografts

A: Mice (n = 8 or 10) were treated with an i.v. injection dose of vehicle control (PBS) or either 2×10^9 pfu or 3.75×10^9 pfu of the indicated viruses administered as a daily regimen of 4×10^8 or 7.5×10^8 pfu, respectively, for five consecutive days on days 1 through 5 of the study. The cumulative fraction of mice surviving over time is plotted. The observed number of complete responses for each cohort is indicated in the legend immediately following the daily dose. The three ONYX-411 cohorts were from three independent studies; those viruses above the dotted line were all done within the same study, whereas the two cohorts of ONYX-411 below the dotted line were from two separate studies. B: Tumors were isolated from mice dosed at 2×10^9 pfu in A on day 71 (dl312 and dl309) and day 65 (ONYX-411) of the study and examined for the presence of newly replicated adenoviral DNA by in situ hybridization. Virally infected tumor cells (dark blue stain; black arrow) are evident throughout the sections from mice infected with either dl309 or ONYX-411.

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Discussion